Measurement of SMN protein in Blood samples Biomarker test for SMA

Thesis

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Abstract

Spinal muscular atrophy (SMA) is a severe motor neuron disease that is the leading genetic cause for infant mortality. This disease arises from mutations in the Survival of Motor Neuron (SMN) gene that are implicated in reducing the overall expression of SMN. Drug development in the SMA field has focused on discovering therapeutic agents that counter these mutations by increasing the expression of SMN. Valproic acid (VPA), a Histone Deacetylase inhibitor, was identified to increase SMN expression in cell culture. However, a recent clinical trial suggested that VPA has no effect on motor function in ambulant adult SMA patients. The interpretation of this result depends upon proof that VPA had the desired effect within cellular nuclei in these patients. In this study, blood samples collected during the course of the clinical trial were analyzed to determine the effect of VPA on molecular biomarkers. Using mass spectrometry, ELISA and immunoassay, we measured histone acetylation and the expression of both SMN protein and mRNA. Systematic analyses showed that while the administration of VPA increased histone acetylation and mRNA, it did not increase the expression of SMN protein. Hence we conclude VPA would not be an efficient drug against SMA and the increase in histone acetylation level does not improve motor functions in SMN patients. VPA fails to improve SMN proteins in vivo. However, due to the potential for SMA biomarker tests, the systematic methods used for the study of SMN protein expressions may still be used for future SMA clinical trial design and biological analysis.

Acknowledgments

I would like to express my greatest gratitude to the people who volunteered in this clinical trial and people in Kolb lab for their support and encouragement throughout my project. I am grateful to Dr. Stephen Kolb for her continuous guidance for the project. I also would like to give special thanks to Samantha Renusch, Sean W. Harshman and Michael A. Freitas for their cooperation and support during the project. I would like to thank my parents for their unconditional love, without which I would be unable to pursue my dream in science and medicine.

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Major Field: Chemical and Biomolecular Engineering

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Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by loss of lower motor neurons during early or postnatal development. Caused by mutation of the survival motor neuron (SMN) gene, SMA is the leading inherited cause of infant and childhood mortality. There is a spectrum of SMA disease severity, and patients can be divided into three clinical groups based on the ability to achieve early motor milestones: type I patients never sit independently, type II patients sit, but never stand and walk unaided, and type III patients stand and walk independently at some point during their disease course.[2]

The majority of patients with SMA have a homozygous deletion/mutation in the *SMN1* (survival of motor neurons 1) gene which results in reduced expression levels of full-length SMN protein. In humans, a nearly identical gene, *SMN2* (survival of motor neurons 2), which also produces some amount of full-length SMN proteins, is present at the same locus and differs from *SMN1* by a single nucleotide substitution that promotes the exclusion of exon 7, resulting in a truncated and dysfunctional protein. (See Figure 1) *SMN2* copy number has an important modifying effect on SMA disease severity and there is a clear inverse correlation between *SMN2* copy number and clinical severity of disease in humans and in murine SMA models. Most patients with SMA type I have one or two *SMN2* copies; type II patients have three *SMN2* copies; and type III have three or four *SMN2* copies. The *SMN2* gene, therefore, provides an ideal target for therapeutic intervention, and interest of investigation for drug discovery.[4]

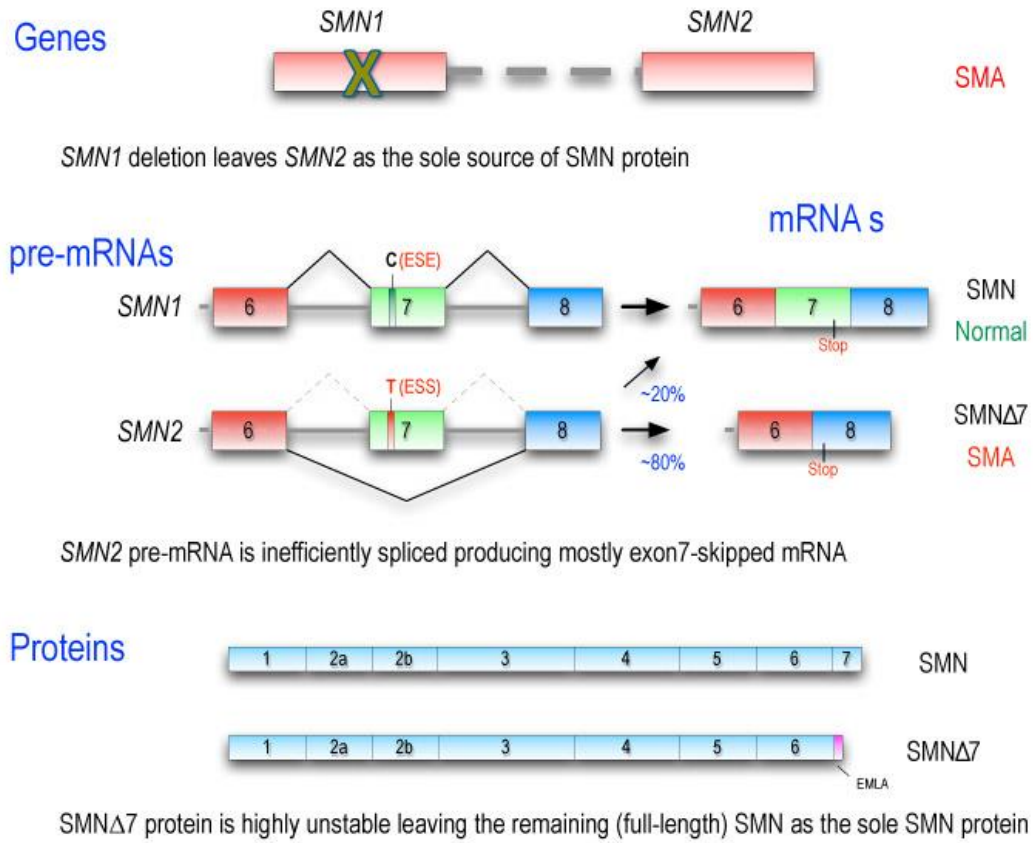


Figure 1: Mechanism of SMN1 and SMN2[3]

Valproic acid (VPA) is one of the first identified molecules that show an effective increase SMN expression levels in vitro and are used in current clinical trials for SMA treatment strategy. It is a histone deacetylase (HDAC) inhibitor that has been shown to increase the expression of full-length SMN protein in patient fibroblasts. VPA treatment was shown to improve gross motor function and the overall number of motor neurons in the spinal cord in treated animals compared to age-matched controls. Past clinical trials of VPA in SMA patients failed to show dramatic improvement of clinical outcome measures. A double blind, randomized, Phase III prospective controlled trial of VPA in ambulant adults with SMA (VALIANT) clinical trial has been completed and found no improvement in the primary clinical outcome measure.

A biomarker, or biological marker, is in general a measurement used as an indicator of a biological state. It is an objectively measured and evaluated to indicate a normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. In our case, changes in SMN expression levels with a clinically meaningful outcome measure can be used to assess and establish the validity of SMN mRNA biomarker. Therefore the SMN expression levels were measured in patients with SMA to investigate whether there is adequate delivery, expected biologic activity, and efficacy of VPA in vivo to test the biomarker.

During the VALIANT trial, multiple blood samples from the adult subjects were obtained and stored for use in molecular SMA biomarker experiments that are hereto proposed.

Meterials and Methods

VALIANT trial design:

Understanding the molecular events that occurred during a clinical trial is always essential for the proper interpretation of the clinical results. My principle of investigator, Dr. Stephen Kolb was involved in a clinical trial for SMA and has already collected a significant amount of peripheral blood mononuclear cells in SMA patients before and after six months of receiving valproic acid. These are the sources of my research project. This clinical trial was a prospective, randomized, double blind, 12 month study in genetically confirmed subjects with SMA type III over 18 years old (ages 18-60). Subjects underwent two baseline assessments and then were placed on VPA or placebo in a blinded fashion for the first six months followed by cross over for an additional 6 months (i.e., subjects originally assigned to receive VPA were switched to placebo and vice versa). Patients were evaluated at 3, 6, and 12 months. There were 20 males and 13 females (mean age 37.1); 16 patients were initially placed on VPA and 17 on placebo. Three patients did not complete the study; two due to adverse events not clearly related

to VPA and one simply withdrew. The drug was sent to The Ohio State University Pharmacy for dispensing to the subjects.

	Screening							
	Visit 1	Visit 2	Safety Lab	Visit 3	Visit 4	Safety Lab	Visit 5	Visit 6
Time – from start of drug	- 4 wks	Start		Month 3	Month 6		Month 9	Month 12
Blood Draw: SMN2 Copy†		X						
Blood Draw: DNA		X						
Blood Draw: mRNA		X		X	X		X	X
Blood Draw: Safety Labs*	X	X**	X@	X	X	X@	X	X
Blood Draw: VPA Level ‡	X		x	X	X	X	X	X
Blood Draw: HDAC activity, SMN protein level, snRNP assembly		x			x			x

Table 1: Schedule of study visits

X* Safety labs include VPA level, CBC, diff, and platelet, ALT, AST, GGT, Alkaline phospahtase, bilirubin, amylase, lipase, Na, K, CL, CO2, BUN, creatinine, and carnitine profile.

X** If abnormal on previous visit

X@ Blood draw will not include carnitine level

† If it was not done previously

‡ Two extra draws at month 1 and 7 to allow dose adjustment

Blood draws for SMN mRNA quantification were collected in PAXgene Blood RNA tubes (BD Diagnostics) during visit 2, 3, 4, 5 and 6. Blood draws to determine HDAC activity

and SMN protein levels were collected during visit 2, 4 and 6. The peripheral mononuclear blood cells (PBMCs) were collected in the Kolb Lab.

Histone:

VPA is hypothesized to act via its ability to inhibit HDACs in order to increase SMN levels. The changes in the acetylation pattern of histones in circulating PBMCs in these subjects were confirmed to interpret the clinical outcome measure results in the VALIANT trial. Cells were lysed in lysis buffer supplemented with inhibitors. Nuclei were extracted and the pellet went through High Pressure Liquid Chromatography before signaled to mass spectrometry. Histone H4 level was recorded. 11349 m/z intensity and area and peak 11349/11307 area ratio and intensity ratio were analyzed and compared for all the post-translational modifications of the major histones in VALIANT subjects.

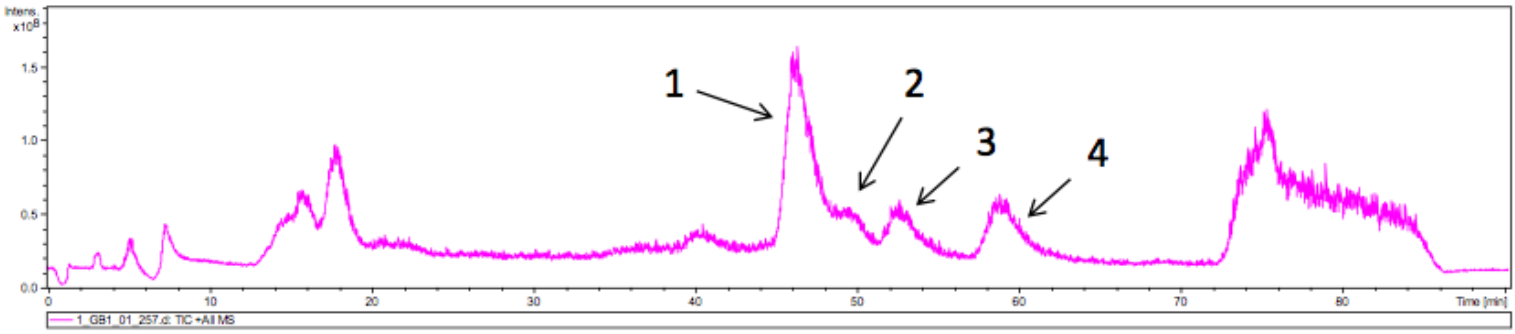


Figure 2: Histone H4 in mass spectrometry

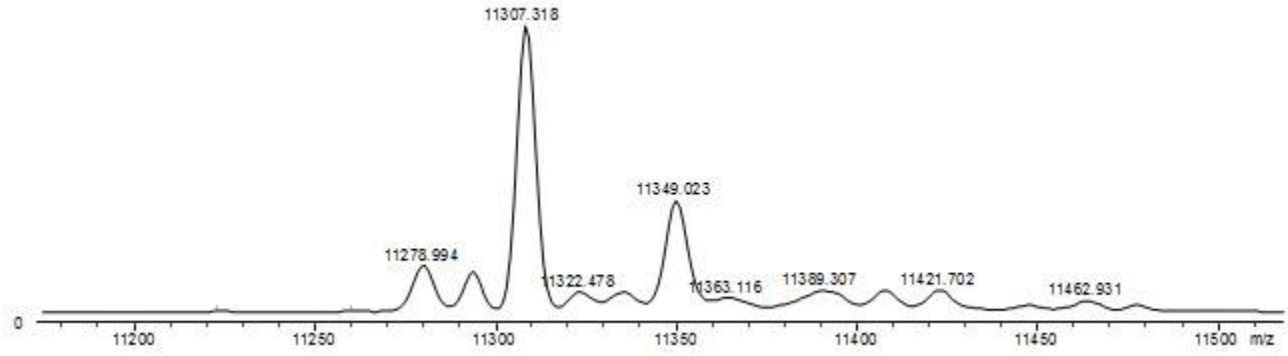


Figure 3: m/z 11307 and 11349

SMN mRNA:

In therapeutic strategies targeting the *SMN2* gene, measures of SMN mRNA can be used to test the effectiveness of a drug and a number of reliable assays have been reported for relative quantification of full-length (SMN-fl) and delta7 (SMN-del7) transcripts in peripheral blood samples. In the absence of drug, SMN expression was very stable over time. In the presence of drug, SMN expression was unaltered or changed in only about one-third of patients. This suggests that any given cohort may comprise both responders and non-responders. There are two major flaws of relative quantification that had caused the inability to distinguish between these responders and non-responders: expression of endogenous controls, used to normalize each reaction for the amount of input RNA and efficacy of RT-PCR, tend to be much more variable within and between patients than SMN expression and their genes may also be affected by drug.[1] Absolute quantification of SMN transcripts is now considered the gold standard and an assay to measure full-length transcripts from the SMN1 and SMN2 genes has now been published.

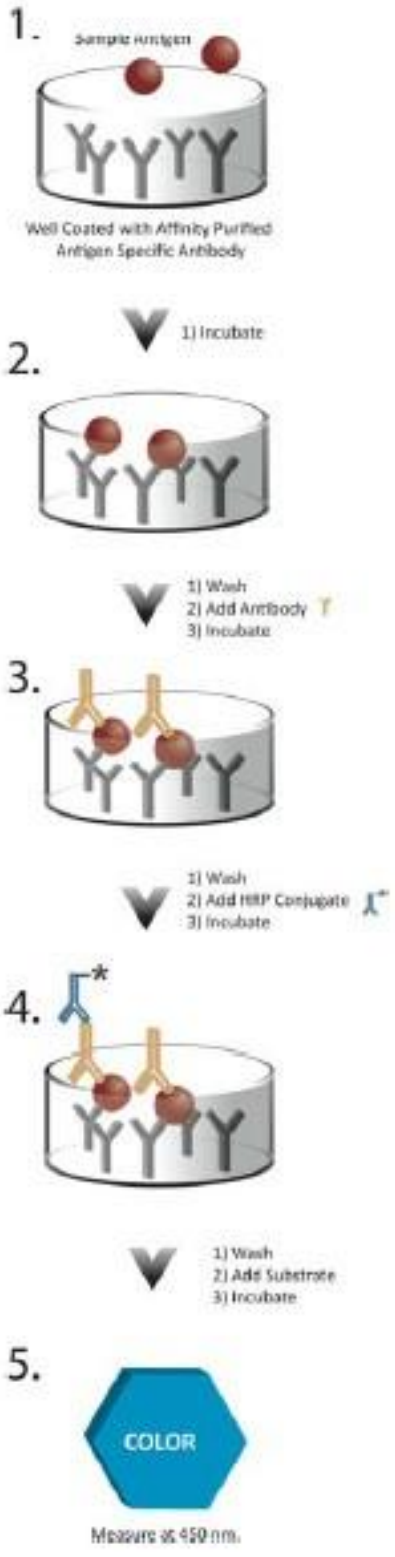
SMN Protein:

Methods for determining protein levels from peripheral blood mononuclear cells (PBMCs) in SMA patients have been reported, however there is no clear consensus as to which method provides the best sensitivity, specificity and feasibility. While mRNA levels are helpful, they do not necessary reflect the amount of protein produced. Studies with VPA and other HDAC inhibitors indicated that changes in SMN protein levels were higher than that observed for SMN mRNA suggesting that protein measures are likely to provide a more sensitive assessment of the extent of drug response. Western blot analysis has been the standard technique for quantification of SMN protein; however there are physical limitations when large numbers of samples are to be compared. In response to these limitations, SMN protein determination by a

cell-based immunoassay and a number of SMN-sensitive two-site ELISAs have been described which correlate with Western Blot outputs.

The quantification of SMN levels from peripheral blood samples was determined using immunoassay; western blot and Enzyme-linked immunosorbent assay (ELISA). Usually western blot analysis is the standard technique for quantification of SMN protein but its use is limited to small sample size. In this case, the cell-based immunoassay and SMN-sensitive two-site ELISAs were used together to determine SMN protein expression.[5] The cell-based immunoassay employs a single monoclonal antibody while ELISA employs two monoclonal antibodies. The immunoassay proved to be unreliable and insensitive in terms of estimation of SMN protein levels. Since the ELISA format was also a more commercially available approach compared to immunoassay, the quantification of SMN protein was determined solely by the two-site ELISA.

Principle of the Two-Site ELISA



1. Samples and standards were added to wells coated with a monoclonal antibody specific for SMN. The plate was then incubated.

2. The plate was washed, leaving only bound SMN on the plate. A yellow solution of rabbit polyclonal antibody to human SMN was then added. It binds to the SMN captured on the plate. The plate was then incubated again.

3. The plate was washed to remove excess antibody. A blue solution of HRP conjugate was added to each well that binds to the rabbit polyclonal SMN antibody. The plate was again incubated.

4. The plate was washed to remove excess HRP conjugate. TMB Substrate solution is added. An HRP-catalyzed reaction generated a blue color in the solution.

5. Stop solution was added to stop the substrate reaction. The resulting yellow color was read at 450 nm. The amount of signal is directly proportional to the level of SMN in the sample.

Figure 4: Principle of two-site Elisa[6]

Results

Mass spectrometry data show that SMA patients treated with valproic acid have a significant increase in histone acetylation levels. The changes in the acetylation state of all major histones isolated from PBMCs correlated to the time when the patients received VPA. The 16 patients who got VPA at the start of the study had high histone acetylation level at the 6-month visit; and the acetylation level went down when they switched to placebo at visit 4. The opposite happened to the 17 patients who received VPA at the 6-month point (visit 4). Their histone acetylation level was high at the end of the year (visit 6). The following graphs are all the patients who completed all the three blood draws.

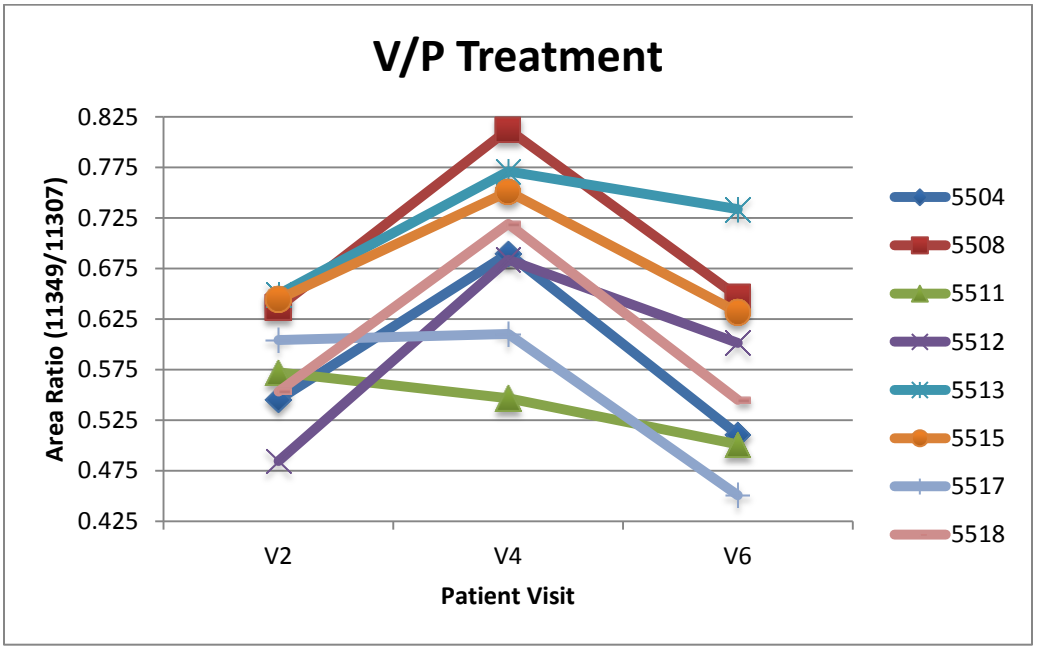


Figure 5: Histone acetylation level for V/P treatment

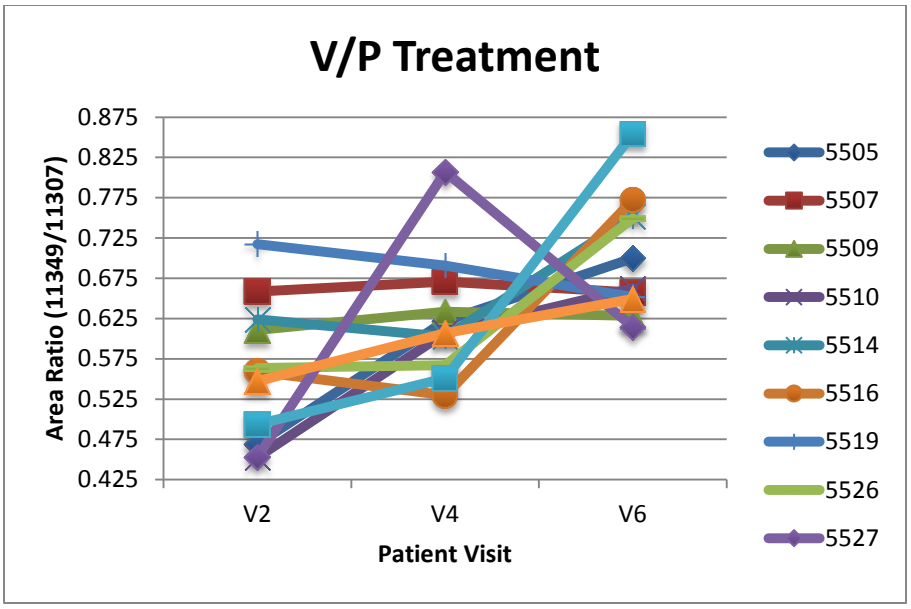


Figure 6: Histone acetylation level for P/V treatment

Patient #5504, 08, 11, 12, 13, 15, 17 and 18 received VPA at V2 and switched to placebo at V4. Patient #5505, 07, 09, 10, 14, 16, 19, 26, 27, 31 and 32 who received placebo at V2 and switched to VPA at V4.

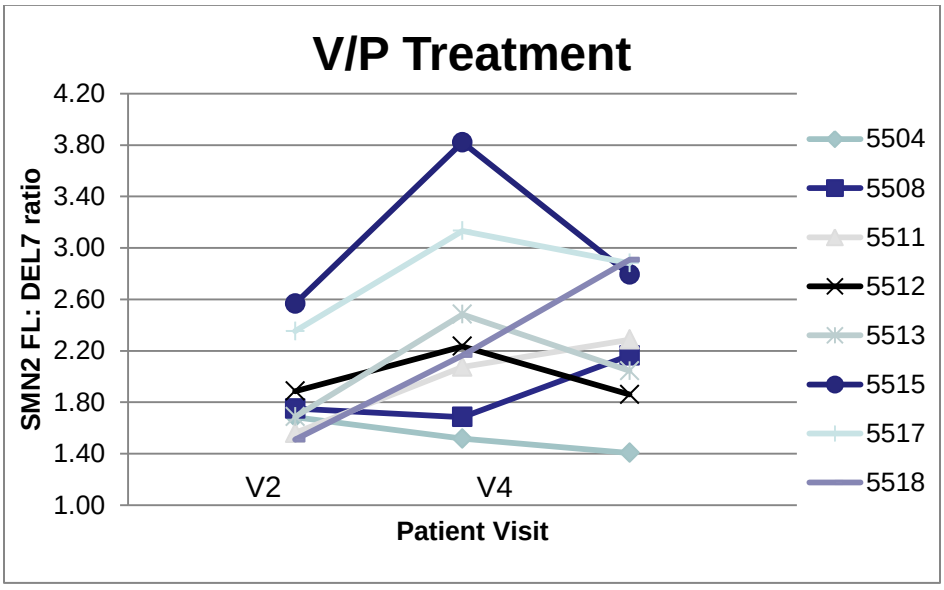


Figure 7: mRNA level for V/P treatment

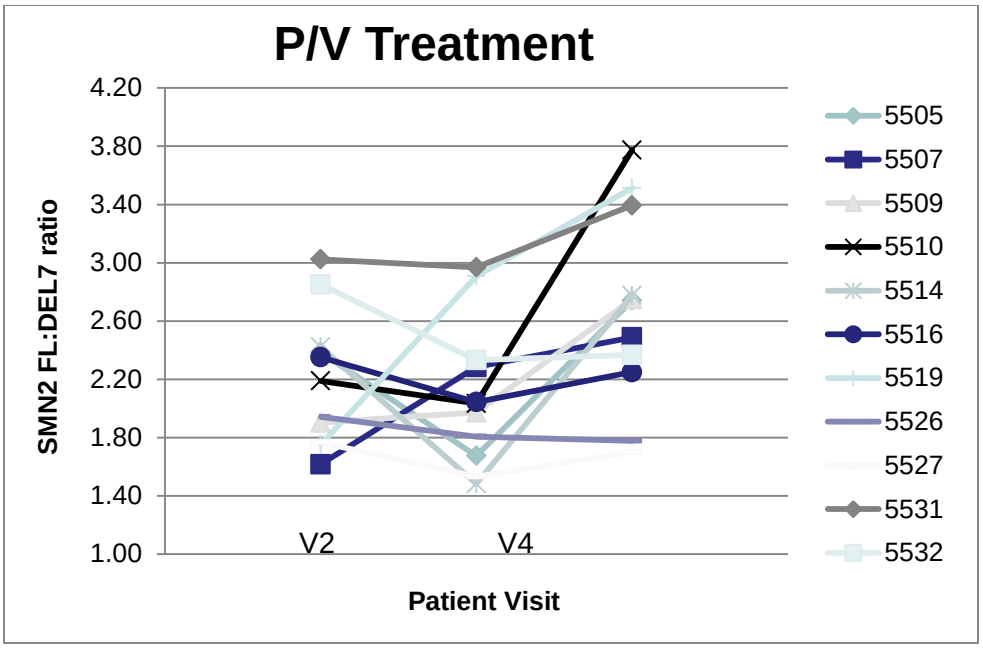


Figure 8: mRNA level for P/V treatment

For the SMN mRNA level, the ratio of the full-length and exon 7-skipped mRNA was recorded. The administration of VPA showed a clear correlation with the FL: Del7 ratio. Patients who took VPA first then switched to placebo had more full-length mRNA for the first half of the trial than the second half, vice versa. VPA had the expected effects on mRNA due to the increase in histone acetylation.

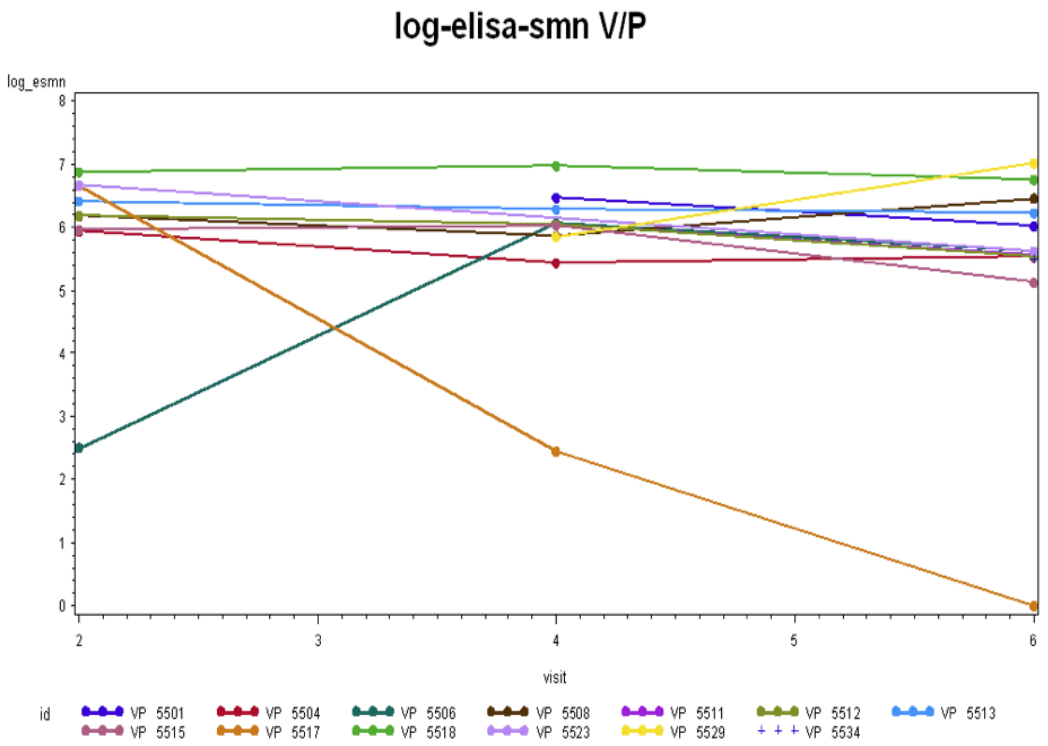


Figure 9: SMN protein level for V/P treatment

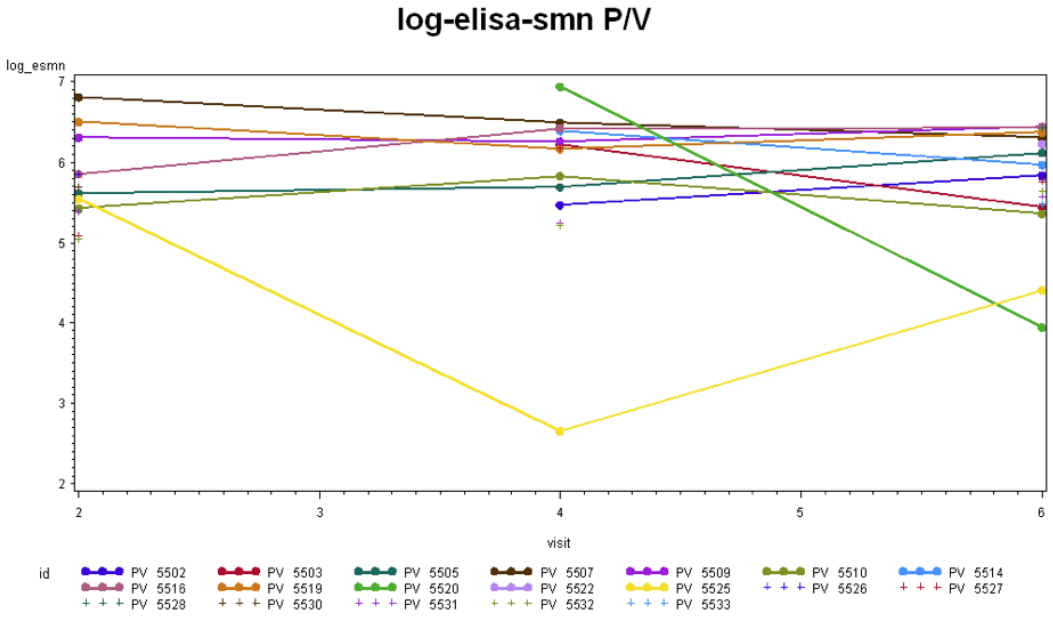


Figure 10: SMN protein level for P/V treatment

The SMN protein level was expected to increase with the treatment of VPA, following a similar pattern with the histone acetylation and mRNA levels; but the results turned out inconclusive. VPA failed to have any molecular effect on the SMN protein level.

Discussion

A key point of the research project is the histone modification analysis of the HDAC inhibitors that are capable of altering histone post-translational modifications. Since there is no change in SMN protein or activity and yet that VPA altered histone acetylation, the conclusion is that VPA had its intended effect peripherally, but failed to increase SMN levels. The lack of the clinical outcome measures may be due to the fact that SMN levels were not increased systemically. In addition, there is a no correlation between clinical outcome measure changes and SMN expression level changes in this tiral.

The expression level of SMN in peripheral blood mononuclear cells (PBMCs) in SMA patients before and after 6 months of VPA administration was measured and compared to determine whether blood SMN protein expression levels can be used as a biomarker for clinical study of SMA. Although the SMN mRNA level increased with the administration of VPA, the SMN protein level was not changed in the subjects' peripheral blood mononuclear cells (PBMCs), biochemistry methods failed to detect any change in molecular function and expression of the SMN proteins, which cannot be used as molecular SMA biomarkers.

The design of therapeutic clinical trials for SMA patients depends on whether or not there is any improvement or better survival chance for the patients. These benefits are fundamentally important if they are to occur. Therefore, there is a need for molecular surrogate assays to determine whether SMN levels are affected in patients that receive such treatments. In this

project, biological samples that were obtained during the course of a clinical trial were analyzed and the SMN expression was measured using validated assays in these well-characterized samples from an adult SMA population. These samples are a unique and valuable resource. The results of this study contribute to the study of SMA disease.

By performing the most thorough analysis of the effect of VPA on histone acetylation in SMA patients, the intended molecular effect of VPA actually occurred systemically, that is, VPA increased histone acetylation level. Since the biochemical analysis is novel, the methods used in this study can be modeled and developed for future SMA therapeutic trials with other agents, contributing to the design of future SMA clinical trials. The protein assay was not more sensitive and reliable than the current mRNA studies in clinical trials. For future design of biological measures in SMA trials, SMN protein level is still not recommended.

Citations

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